PCT / CA 99/00314
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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,234,588**, on April 7, 1998, by **THE SIR MORTIMER B. DAVIS - JEWISH GENERAL HOSPITAL**, assignee of John Hiscott and Rongtuan Lin, for "Highly Active Forms of Interferon".

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VIRUS INDUCED PHOSPHORYLATION OF INTERFERON REGULATORY FACTOR-3

BACKGROUND OF THE INVENTION

Interferons (IFNs) are a large family of multifunctional secreted proteins involved in antiviral defence, cell growth regulation and immune activation (63). Virus infection induces the transcription and synthesis of multiple IFN genes (33,52,63); newly synthesized IFN interacts with neighbouring cells through cell surface receptors and the JAK-STAT signalling pathway, resulting in the induction of over 30 new cellular proteins that mediate the diverse functions of the IFNs (17,35,39,58). Among the many virus- and IFN-inducible proteins are the growing family of IRF transcription factors, the Interferon Regulatory Factors (IRFs).

IRF-1 and IRF-2 are the best characterized members of this family, originally identified by studies of the transcriptional regulation of the human IFN-β gene (22,23,30,47). Their discovery preceded the recent expansion of this group of IFN-responsive proteins which now include seven other members: IRF-3, IRF-4/Pip/ICSAT, IRF-5, IRF-6, IRF-7, ISGF3γ/p48 and ICSBP (48). Structurally, the Myb oncoproteins share homology with the IRF family, although its relationship to the IFN system is unclear (62). Recent evidence also demonstrates the presence of virally encoded analogue of cellular IRFs - vIRF in the genome of human herpes virus 8 (HHV8) (55).

The presence of IRF-like binding sites in the promoter region of the IFNA and IFNB genes implicated the IRF factors as essential mediators of the induction of IFN genes. The original results of Harada et al. (30,32) indicated that IFN gene induction was activated by IRF-1, while the related IRF-2 factor suppressed IFN expression. However, the essential role of IRF-1 and IRF-2 in the regulation of IFNA and IFNB gene expression has become controversial with the observation that mice containing homozygous deletion of IRF-1 or IRF-2, or fibroblasts derived from these mice, induced IFNA and IFNB gene expression after virus infection to the same level as the wild-type mice or cells (44).

On the other hand, IRF-1 was shown to have an important role in the antiviral effects of IFNs (44,54). IRF-1 binds to the ISRE element present in many IFN-inducible gene promoters and activates expression of some of these genes (54). However, activation of ISG genes by IFNA and IFNB was shown to be mediated generally by the multiprotein ISGF3 complex (31,36,38). The binding of this complex to DNA is mediated by another member of the IRF family, ISGF3γ/p48, which in IFN-treated cells interacts with phosphorylated STAT1 and STAT2 transcription factors forming the heterotrimeric complex ISGF3 (8,39,62). The homozygous deletion of p48 in mice abolished the sensitivity of these mice to the antiviral effects of IFNs, and virus-infected macrophages from p48-/- mice showed an impaired induction of IFNA and IFNB genes (31).

Several other members of the IRF family have been identified. The ICSBP gene is expressed exclusively in the cells of the immune system (18,64) and its expression can be enhanced by IFNγ. ICSBP was shown to form a complex with IRF-1 and inhibit the transactivating activity of IRF-1 (9,59). The homozygous deletion of ICSBP in mice leads to defects in myeloid cell lineage development and chronic myelogous leukemia (34). Another lymphoid specific Pip/LSIRF/IRF-4 was identified (19,43,66) that interacts with phosphorylated PU.1, a member of the Ets family of transcription factors (15). The Pip/PU.1 heterodimer can bind to the immunoglobulin light chain enhancer and function as a B cell specific transcriptional activator. Expression of Pip/LSIRF was induced by antigenic stimulation but not by IFN, and Pip/LSIRF/IRF-4 -/- mice failed to develop mature T and B cells (46). A novel member of the IRF family was recently identified by its ability to bind to an ISRE-like element in the promoter region of the Qp gene of EBV (69).

Another unique member of the human IRF family, IRF-3 was characterized recently (2). The IRF-3 gene encodes a 55-kDa protein which is expressed constitutively in all tissues. Recombinant IRF-3 binds to the ISRE element of the IFN-induced gene ISG-15 and stimulates this promoter in transient expression assays. In contrast to IRF-1, which contains both DNA binding and transactivating domains, IRF-3 - like IRF-2 and ICSBP - does not contain a well-defined transactivation domain. Viral induction or IFN-treatment does not stimulate the expression of the IRF-3 gene. In

previous studies, applicant showed that IRF-3 binds to the IE and PRDIII regions of the IFNA and IFNB promoters respectively, but has different effects on their transcriptional activity (56). While the induction of the IFNA4 promoter activated by IRF-1 or virus infection was inhibited in the presence of IRF-3, the fusion protein containing the IRF-3 DNA binding domain and the RelA(p65) transactivation domain effectively activated both IFNA and IFNB promoters. In contrast, co-expression of IRF-3 and RelA plasmids transactivated the IFNB gene promoter, but not the promoter of the IFNA4 gene (56).

The applicant has examined the virus-induced, post-translational modulation of IRF-3 protein following Sendai virus infection. Applicant's studies demonstrate that phosphorylation represents an important post-translational modification of IRF-3 leading to cytoplasmic to nuclear translocation of phosphorylated IRF-3, stimulation of DNA binding and transcriptional activity, association of IRF-3 with the transcriptional co-activator CBP/p300, and ultimately proteasome mediated degradation.

SUMMARY OF THE INVENTION

The interferon regulatory factors (IRF) consist of a growing family of related transcription proteins first identified as regulators of the IFNα/β gene promoters, as well as the ISRE of some IFN-stimulated genes. IRF-3 was originally identified as a member of the IRF family based on homology with other IRF family members and on binding to the ISRE of the ISG15 promoter. IRF-3 is expressed

constitutively in a variety of tissues and the relative levels of IRF-3 mRNA do not change in virus-infected or IFN-treated cells.

Applicant has demonstrated that, following Sendai virus infection, IRF-3 is post-translationally modified by protein phosphorylation at multiple serine and threonine residues, located in the carboxy-terminus of IRF-3. A combination of IRF-3 deletion and point mutations localized the inducible phosphorylation sites to the region -ISNSHPLSLTSDQ- between amino acids 395 and 407; point mutation of Ser-396 and Ser-398 residues eliminated virus-induced phosphorylation of IRF-3 protein, although residues Ser-402, Thr-404 and Ser-405 were also targets. Phosphorylation results in the cytoplasmic to nuclear translocation of IRF-3, DNA binding and increased transcriptional activation. Substitution of the Ser/Thr sites with the phosphomimetic Asp generated a constitutively active form of IRF-3 that functioned as a very strong activator of promoters containing PRDI/PRDIII or ISRE regulatory elements. Use of phosphomimetic Glu for this purpose is also possible. Phosphorylation also appears to represent a signal for virus mediated degradation, since the virus induced turnover of IRF-3 was prevented by mutation of the IRF-3 Ser/Thr cluster or by proteasome inhibitors.

Interestingly, virus infection resulted in the association of IRF-3 with the CBP coactivator, as detected by co-immunoprecipitation with anti-CBP antibody, an interaction mediated by the C-terminal domains of both proteins.

Mutation of the residues Ser-396 and Ser-398 in IRF-3 abrogated its binding to CBP. These results are discussed in terms of a model in which virus-inducible C-terminal phosphorylation of IRF-3 alters protein conformation to permit nuclear translocation, association with transcriptional partners and primary activation of IFN- and IFN-responsive genes.

DETAILED DESCRIPTION OF THE INVENTION

Several novel aspects of post-translational regulation of the IRF-3 transcription factor are highlighted in the present study. 1) Sendai virus dependent phosphorylation of IRF-3 was detected, occurring in a cluster of Ser and Thr sites in the carboxyl-terminal end of the protein. The residues implicated in this regulatory phosphorylation event are Ser-396/Ser-398/Ser-402/Thr-404/Ser-405, particularly the Ser-396/Ser-398 amino acids. 2) Phosphorylation of the IRF-3 in the Ser-Thr cluster resulted in the cytoplasmic to nuclear translocation of IRF-3; nuclear translocation was blocked by mutation of the phosphorylated amino acids. 3) Sendai virus infection induced the DNA binding and transactivation potential of IRF-3. Furthermore, IRF-3 containing the phosphomimetic Asp at the sites of C-terminal phosphorylation was an exceptionally strong transactivator of PRDI/PRDIII and ISRE containing promoters. 4) Phosphorylation was also required for the association of IRF-3 with the CBP co-activator protein. 5) Sendai virus infection resulted in IRF-3 degradation; again, phosphorylation was required as a

signal for inducer-mediated degradation since mutation of Ser/Thr cluster also blocked virus induced degradation.

Cytoplasmic to nuclear translocation of IRF-3 as a consequence of virus infection was inhibited by mutation of the Ser/Thr cluster, indicating an important regulatory role for C-terminal phosphorylation in the activation of IRF-3. Also strikingly, the conversion of the phosphorylation sites to the phosphomimetic Asp altered the subcellular localization of IRF-3 in uninfected cells. A proportion of IRF-3(5D) was localized to the nucleus of uninfected cells, suggesting that some IRF-3 may shuttle to and from the nucleus constitutively; this observation is consistent with the identification of a nuclear export signal in IRF-3. Mutation of L144A/L145A in the NES element produced the most impressive alterations in subcellular localization. In uninfected cells, IRF-3 was partitioned in both the nucleus and cytoplasm; virus infection changed the nuclear pattern of staining from extra-nucleolar homogeneous staining as observed for wtIRF-3 to an intense nuclear speckling. At this stage, the nature of the subnuclear changes in IRF-3 localization are not explained, although it is possible that IRF-3(NES) translocates efficiently into the nucleus but becomes trapped in the nuclear pore complex during the export process.

One of the striking results of the mutagenesis of the C-terminal domain of IRF-3 was the generation of IRF-3(5D), an exceptionally strong activator of IFN-β and ISG-15 gene expression. The phosphomimetic form of IRF-3

alone was able to stimulate IFN-β expression as strongly as virus infection, a level of stimulation not previously observed in co-expression experiments (24,61). In previous experiments, applicant demonstrated that IRF-3 was able to bind the ISRE element of ISG-15, as well as the PRDIII/PRDI and IE regions of the IFNB and IFNA promoters, respectively (2,56). Virus induction results in the appearance of two new protein-DNA complexes; supershift experiments confirmed that both complexes contain IRF-3; it is not clear at this stage whether the upper complex also contains other proteins such as in the VIC (10,29) and DRAF (16) complexes or whether the lower complex represents a breakdown product of IRF-3. Strikingly, the same complexes appeared following co-transfection of IRF-3(5D) expression plasmid in the absence of virus induction, indicating that IRF-3(5D) represented a constitutive DNA binding form of IRF-3. Thus, in uninfected cells, IRF-3(5D) localized in part to the nucleus (Fig. 5), interacted with DNA constitutively and was a strong activator of gene expression (Fig. 6).

The recent crystal structure of the related IRF-1 protein bound to PRDI provides evidence for a novel helix-turn-helix motif that latches onto a GAAA core sequence via three of the five conserved tryptophan amino acids of the DNA binding domain (20). By analogy with IRF-3, two GAAANN sequences present in PRDIII of IFN-β and another GAAANN element present in PRDI may serve as DNA contacts for multiple IRF-3(5D) proteins with strong activating potential. Similarly, the ISRE element of the ISG-15 promoter also

contains several GAAANN anchors for potential IRF binding. Given the range of promoters that possess this hexameric sequence (48), it will be of interest to determine the capacity of IRF-3(5D) to stimulate expression of different cytokine and chemokine genes.

IRF-3 joins a growing list of cellular and viral proteins that functionally interact with CBP/p300 proteins, highly homologous proteins originally identified through their interactions with adenovirus E1A and CREB proteins (1,13). As a critical determinant of its global transcriptional coactivator activity, CBP/p300 possesses histone acetyltransferase activity (5,50). Acetylation of histones is involved in the destabilization and remodelling of nucleosomes, a crucial step in permitting the accessibility of transcriptional factors to DNA templates. Several studies have now demonstrated that CBP/p300 participates in the transcriptional process by providing a scaffold for different classes of transcriptional regulators on specific chromatin domains (12,50). A growing body of biochemical and genetic evidence also implicates CBP/p300 as a negative regulator of cell growth, based on its interactions with adenovirus E1a, SV40 large T antigen and the tumour suppressor p53, among others. With regard to p53-CBP/p300 complex formation, functional interaction between these two important growth regulatory proteins accounts for several of the known activities of p53 (3,28,40); interestingly, CBP/p300 was shown recently to acetylate p53 and stimulate its transactivation potential

(27).

It will be of interest to determine whether IRF-3 is similarly modified by CBP association. The functional consequences of IRF-3 interaction with CBP/p300 remain to be elucidated, although recent studies demonstrated that CBP/p300 also functionally interacts with STAT 1 (68) and STAT 2 (7) and may contribute to IFNα and IFNγ nuclear signalling. Recently published studies have demonstrated that synergistic activation of the IFNβ promoter requires recruitment of CBP/p300 to the enhanceosome, via a new activating surface assembled from the activation domains of all the transcription factors in the enhanceosome (37,45). Alterations in any of the activation domains decreased both CBP recruitment and transcriptional synergy. By analogy, recruitment of CBP/p300 to DNA bound IRF-3 is likely required for maximal transcriptional activation. Association requires the interaction of the C-terminal domain of IRF-3 and the C-terminal interaction domain of CBP, a region previously shown to associate with the p53 tumour suppressor, whereas STAT1 and STAT2 associate with different regions of CBP (7,68).

Virus induced phosphorylation of IRF-3 also represents a signal for proteasome mediated degradation of IRF-3, since mutation of the Ser-396/Ser-398 or the use of proteasome inhibitors prevented the post infection degradation of IRF-3. Virus induced degradation of IRF-3 is reminiscent of the virus-induced turnover of another member of the IRF family - IRF-2. In response to dsRNA or viral

induction, the 50 kD IRF-2 protein is proteolytically processed into a smaller, 24-27 kDa protein (51) comprising the 160 aa DBD of IRF-2, termed TH3 (14) or In4 (65). Although TH3 has been shown to bind DNA and repress transcription more efficiently than the full length IRF-2 protein (42), its physiological role is not clear. Since the induction kinetics of TH3 are slower than that of IFN-β in response to dsRNA or viral infection (14), it has been suggested that the IRF-2 cleavage product may be a post-induction repressor of IFN-β gene expression (65).

Virus induced phosphorylation of IRF-3 at the C-terminal Ser/Thr residues and its subsequent degradation by a proteasome dependent pathway are also similar to the well studied phosphorylation and degradation of IκBα which leads to activation of NF-κB binding activity (reviewed in 4,6). In unstimulated cells, NF-κB heterodimers are retained in the cytoplasm by inhibitory IκB proteins. Upon stimulation by many activating agents, including cytokines, viruses and dsRNA, IκBα is rapidly phosphorylated and degraded, resulting in the release and nuclear translocation of NF-κB. The amino-terminus of IκBα represents a signal response domain for activation of NF-κB and substitution of alanine for either Ser-32 or Ser-36 completely abolished the signal-induced phosphorylation and degradation of IκBα, and blocked the activation of NF-κB. These mutations also blocked *in vitro* ubiquitination of the IκBα protein. The amino-terminus of IκBα is necessary for signal-induced phosphorylation and ubiquitination, but for degradation to

occur, there is an absolute requirement for the C-terminal PEST domain (reviewed in 4,6).

Similarities and differences exist between the observed degradation of IRF-3 and the mechanism of IκBα degradation. The C-terminal phosphorylation of IRF-3 as a consequence of virus infection is required for its subsequent degradation based on the deletion and point mutation analysis of the region -ISNSHPLSLTSDQ- between amino acids 395 and 407. Minimally, phosphorylation of Ser-396 and Ser-398 are required for subsequent degradation, although Ser-402, Ser-404 and Ser-405 may represent secondary phosphorylation sites. Likewise, in the case of IκBα, phosphorylation and Ser-32 and Ser-36 are required for inducer mediated degradation. Furthermore, the protease inhibitor calpain inhibitor I and the more specific proteasome inhibitor MG132 block IRF-3 turnover.

A major difference in the mechanisms of IκBα and IRF-3 turnover lies in the nature of the inducing stimuli. Multiple inducers - cytokines such as TNF and IL-1, viruses, LPS, oxidative stress, etc (6) - all lead to the induction of IκBα phosphorylation and degradation whereas IRF-3 phosphorylation appears to be induced only by virus infection and dsRNA addition; other inducers have not resulted in IRF-3 turnover.

A significant temporal difference also exists between IκBα phosphorylation/turnover and IRF-3 phosphorylation/degradation. Many activators of NF-κB stimulate IκBα phosphorylation within minutes and TNF induced

degradation occurs within the first 15-30 min after treatment. In the case of IRF-3, phosphorylation is not detected until 6-8 hours after infection and continues in a heterogenous manner over the next 10-12 hours. Previous experiments have, however, demonstrated that Sendai virus-induced turnover of IκBα also occurs slowly over several hours (24).

Based on the data presented herein and by analogy with the properties of other IRF family members (48), applicant proposes the following model to explain several observations. IRF-3 exists in a latent state in the cytoplasm of uninfected cells; the C-terminus may physically interact with the DNA binding domain in such a way as to obscure both the DBD and the IAD regions of the protein; the presence of an autoinhibitory domain within the C-terminal 20aa (407-427) would explain the activating effect of this deletion, as seen previously with IRF-4 (11,19). Virus induced phosphorylation at the Ser/Thr at 396-405aa cluster leads to a conformational change in IRF-3, exposing both the DBD and IAD and relieving C-terminal autoinhibition. Translocation to the nucleus, occurring via an unidentified nuclear localization sequence or in conjunction with a transcriptional partner associating through the IAD region, leads to DNA binding at ISRE- and PRDI/PRDIII-containing promoters. Phosphorylation is also necessary for IRF-3 association with the chromatin remodelling activity of CBP/p300. The presence of a NES element ultimately shuttles IRF-3 from the nucleus and terminates the initial activation

of IFN responsive promoters. The phosphorylated form of IRF-3 exported from the nucleus may now be susceptible to proteasome mediated degradation. This scenario shares several features with the protein synthesis independent activation of NF-κB, and further suggests that IRF-3 may represent a component of virus- or dsRNA-inducible complexes such as DRAF (16) or VIC (10,29) that could play a primary role in the induction of IFN- or IFN responsive genes.

DESCRIPTION OF THE FIGURES

Figure 1. Sendai virus infection induces IRF-3 degradation.

IRF-3 expression plasmid CMVBL-IRF3 (lanes 1 and 2) or CMVBL vector alone (lanes 3 and 4), both at 5 μg were transiently transfected into 293 cells by the calcium phosphate method. At 24h post transfection, cells were infected with Sendai virus for 16h (lanes 2 and 4) or left uninfected (lanes 1 and 3). Whole cell extracts (20 μg) were prepared and analyzed by immunoblotting with anti-IRF-3 antibody.

Figure 2. Sendai virus induced phosphorylation and degradation of IRF-3 protein.

A) rtTA-IRF-3 cells, selected as described in the Example, were induced to express IRF-3 by doxycycline treatment for 24h. At 24h after Dox addition, cells were infected with Sendai virus for 4, 8, 12, 16, 20, or 24h (lanes 2-7) or were left uninfected (lane 1). IRF-3 protein was detected in whole cell extracts (10 μg) by immunoblot. Two forms of IRF-3 were detected, designated as form I and form II.

B) At 24h post Dox induction, rtTA-IRF-3 cells were infected

with Sendai virus for 16 hours (lanes 4-8) or were left uninfected (lanes 1-3). Whole cell extracts from untreated cells (20 μg) or Sendai virus infected cells (60 μg) were incubated with 0.3 units of potato acidic phosphatase (PPA, lanes 2, 3, 7 and 8) or 5 units of calf intestinal alkaline phosphatase (CIP, lanes 4 and 5) in the absence (lanes 1, 2, 4, 6 and 7) or presence of phosphatase inhibitors (lanes 3, 5 and 8). Phosphorylated IRF-3 protein appears as a distinct band in immunoblots, migrating more slowly than IRF-3 forms I and II.

Figure 3. Analysis of IRF-3 deletion mutants in Sendai virus induced phosphorylation.
(A) Schematic representation of four IRF-3 deletions. Thick solid lines and thin dashed lines indicate included and excluded sequences, respectively. The N-terminal IRF homology domain, the nuclear export signal (NES) and C-terminal IRF association domain are indicated.
(B) Expression plasmids (5 μg each) encoding wild type and deletion mutants of IRF-3 (as indicated above the lanes) were transiently transfected into 293 cells; at 24h post transfection, cells were infected with Sendai virus for 16h (lanes 2, 4, 6, 8, and 10) or left uninfected (lanes 1, 3, 5, 7, and 9). Whole cell extracts (20 μg) were prepared from infected and control cells and analyzed by immunoblotting for IRF-3 forms I and II and for the presence of phosphorylated IRF-3 (P-IRF-3) with anti-IRF-3 antibody.

Figure 4. Analysis of IRF-3 point mutations in Sendai virus induced phosphorylation.

(A) Schematic representation of IRF-3 point mutations. Thick solid lines and thin dashed lines indicate included and excluded sequences, respectively. The N-terminal IRF homology domain, the Nes element and C-terminal IRF association domain are indicated. Amino acids residues from 382 to 414 and from 141 to 147 are shown. The amino acids targeted for alanine or aspartic acid substitution are shown in large print. The point mutations are indicated below the sequence: (2A: S396A/S398A; 3A: S402A/T404A/S405A; 5A: S396A/S398A/S402A/T404A/S405A); 5D S396D/S398D/S402D/T404D/S405D; J2A: S385A/S386A; NES: S145A/S146A).

(B) Expression plasmids (5 μg each) encoding wild type and point mutants of IRF-3 (as indicated above the lanes) were transiently transfected into 293 cells; at 24h post transfection, cells were infected with Sendai virus for 16h (lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18) or left uninfected (lanes 1, 3, 5, 7, 9, 11, 13, 15 and 17). Whole cell extracts (20 μg) were prepared from infected and control cells and analyzed by immunoblotting for IRF-3 forms I and II and for the presence of phosphorylated IRF-3 (P-IRF-3) with anti-IRF-3 antibody.

Figure 5. Virus dependent cytoplasmic-nuclear translocation of IRF-3.

The subcellular localization of the GFP-IRF-3 (A and B), GFP-IRF-3(5A) (C and D), GFP-IRF-3(5D) (E and F) and GFP-IRF-3(NES) (G and H) was analyzed in uninfected (A, C, E, and G) and Sendai virus infected COS-7 cells at 16h after

infection. GFP fluorescence was analyzed in living cells with a Leica fluorescence microscope using 40x objective.

Figure 6. Transactivation of PRDI/PRDIII and ISRE containing promoters by IRF-3.

293 cells were transfected with IFNβ-CAT (A and B) or ISG15-CAT (C) reporter plasmids and the various expression plasmids as indicated below the bar graph. CAT activity was analyzed at 48h post-transfection with 100 μg (IFNβ-CAT) or 10 μg (ISG15-CAT) of total protein extract for 1-2h at 37°C. Relative CAT activity was measured as fold activation (relative to the basal level of reporter gene in the presence of CMV-Bl vector alone after normalization with co-transfected β-Gal activity); the values represent the average of three experiments with variability shown in the error bar.

Figure 7. Stabilization of IRF-3 by proteasome inhibitors.

IRF-3 ΔN (Δ9-133) (B) or IRF-3 ΔN2A (C) expression plasmids were transiently transfected into 293 cells; at 24h post transfection, cells were infected with Sendai virus and treated for 12h with calpain inhibitor I (100 μM, lanes 2 and 5) or MG132 proteasome inhibitor (40 μM, lanes 3 and 6). Ethanol, the solvent for calpain inhibitor I and MG132, was added to the cells as control (lanes 1 and 4). Endogenous (A) and transfected (B and C) IRF-3 proteins were detected in whole cell extracts (20 μg) by immunoblot.

Figure 8. IRF-3 interacts with CBP in virus infected cells.

(A) Schematic representation of CBP, illustrating the domains involved in interaction with host or viral proteins (modified from (28)) and the myc-tagged CBP proteins (CBP1, CBP2, CBP3) used for immunoprecipitation.

(B) 293 cells were transfected with wild type and deletion mutants of IRF-3 expression plasmid (5 μg, as indicated above the lanes) or left untransfected (lanes 1 and 8). At 24h after transfection, cells were infected with Sendai virus for 16h (lanes 1, 3-8, and 10-13) or left uninfected (lanes 1 and 9). Whole cell extracts (300 μg, except lane 1, which was 600 μg) were immunoprecipitated with anti-CBP antibody A22 (lanes 1-6) or with preimmune serum (lane 7). The immunoprecipitated complexes (lanes 1-7) or 30 μg whole cell extracts (lanes 8-13) were run on 5% SDS-PAGE and subsequently probed with anti-IRF-3 antibody.

(C) 293 cells were co-transfected with myc-tagged CBP expression plasmids (as indicated above the lanes) and IRF-3 ΔN (Δ9-133) expression plasmid. At 24h after transfection, cells were infected with Sendai virus (lanes 2, 4 and 6) or left uninfected (lanes 1, 3 and 5). Whole cell extracts (300 μg) were immunoprecipitated with monoclonal anti-myc-tag antibody 9E10. The immunoprecipitated complexes were run on 5% SDS-PAGE and different forms of IRF-3 in the precipitates were analyzed by immunoblotting with anti-IRF-3 antibody.

(D) Whole cell extracts (30 μg) from (C) were also analyzed directly for the expression of myc-tagged CBP proteins by immunoblotting using anti-myc antibody 9E10.

EXAMPLE

Plasmid constructions and Mutagenesis.

The IRF-3 expression plasmid was prepared by cloning the *Eco*RI-*Xho*I fragment containing the IRF-3 cDNA from the pSKIRF-3 plasmid downstream of the CMV promoter of CMVBL vector. CMVt-IRF-3 was constructed by cloning of IRF-3 cDNA downstream of the doxycycline-responsive promoter CMVt at the *Bam*HI site of the neo CMVt BL vector (49). cDNAs encoding IRF-3 carboxyl terminal deletion mutations were generated by 28 cycles of PCR amplification with Vent DNA polymerase. DNA oligonucleotide primers were synthesized using an Applied Biosystems DNA/RNA synthesizer. The amino-terminal primer was synthesized with an *Eco*RI restriction enzyme site and the carboxyl-terminal primers were synthesized with *Xba*I restriction enzyme sites at their ends. The PCR products were purified by phenol/chloroform extraction and ethanol precipitation, digested with *Eco*RI and *Xba*I, and inserted into *Eco*RI/*Xba*I sites of CMVBL vector.

The point mutations of IRF-3 were generated by overlap PCR mutagenesis using Vent DNA polymerase. Mutations were confirmed by sequencing. The N-terminal deletion mutations (ΔN, ΔN2A, ΔN3A and ΔN5A) of IRF-3 were generated by digestion of the related IRF-3/CMVBL plasmid with *Bam*HI (filled in with Klenow enzyme), partial digestion with *Sca*I, and re-ligation. GFP-IRF-3 expression plasmids were generated by cloning of cDNAs encoding wild type or mutated forms of IRF-3 into the downstream of EGFP in the pEGFP-C1 vector (Clonetech). For construction of plasmids encoding

myc-tagged CBP truncated proteins, the cDNAs coding for CBP were generated from the pRC-RSV/mCBP plasmid (provided by Dr. Dimitris Thanos) by PCR amplification. The cDNA fragments were cloned in the downstream of myc-tag in 5' myc-PCDNA3 vector (provided by Dr. Stephane Richard).

Generation of IRF-3 cell lines.

Plasmid CMVt-rtTA (49) was introduced into 293 cells by a calcium phosphate-based method. Cells were selected beginning at 48h after transfection for about one week in αMEM media (GIBCO-BRL) containing 10% heat-inactivated calf serum, glutamine, antibiotics and 2.5 ng/μl puromycin (Sigma). Resistant cells carrying the CMVt-rtTA plasmid (rtTA-293 cells) were then transfected with the CMVt-IRF-3 plasmid. Cells were selected beginning at 48h for a period of approximately 2 weeks in αMEM containing 10% heat-inactivated calf serum, glutamine, antibiotics, 2.5 ng/μl puromycin and 400 μg/ml G418 (Life Technologies, Inc.).

Cell culture and transfections.

All transfections for CAT assay were carried out in human embryonic kidney 293 cells or NIH3T3 cells grown in αMEM (293) or Dulbecco's MEM (NIH3T3) media (GIBCO-BRL) supplemented with 10% calf serum, glutamine and antibiotics. Subconfluent cells were transfected with 5 μg of CsCl purified chloramphenicol acetyltransferase (CAT) reporter and expression plasmids by calcium phosphate coprecipitation method (293 cells) or lipofectamine (NIH3T3 cells). The reporter plasmids were the SVoβ CAT and ISG15 CAT reporter

genes (56); also the transfection procedures were previously described (41,56). For individual transfections, 100 μg (SVoβ CAT) or 10 μg (ISG15 CAT) of total protein extract was assayed for 1-2h at 37°C. The CAT activity was normalized with β-Gal assay. All transfections were performed 3-6 times.

Western blot analysis of IRF-3 modification and degradation.

To characterize the posttranslational regulation of IRF-3 protein, stable or transiently transfected IRF-3 expressing cells were infected with Sendai Virus (80 HAU/ml) or treated with 5 ng/ml TNF-α, either with or without addition of 50 μg/ml cycloheximide. In some experiments, cells were treated with either 100 μM calpain inhibitor I (ICN), 40 μM MG132 proteasome inhibitor, or an equivalent volume of their respective solvent (ethanol) as control. Cells were washed with phosphate-buffered saline and lysed in 10 mm Tris-Cl pH 8.0, 200 mm NaCl, 1 mm EDTA, 1 mm dithiothreitol (DTT), 0.5% Nonidet P-40 (NP-40), 0.5 mm phenylmethysulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 5 μg/ml aprotinin. Equivalent amounts of whole cell extract (20 μg) were subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% polyacrylamide gel. After electrophoresis, the proteins were transferred to Hybond transfer membrane (Amersham) in a buffer containing 30 mm This, 200 mm glycine and 20% methanol for 1h. The membrane was blocked by incubation in phosphate-buffered saline (PBS) containing 5% dried milk for 1h and then probed with IRF-3 antibody in 5% milk/PBS, at a

dilution of 1:3000. These incubations were done at 4°C overnight or at RT for 1-3h. After four 10 minute washes with PBS, membranes were reacted with a peroxidase-conjugated secondary goat anti-rabbit antibody (Amersham) at a dilution of 1:2500. The reaction was then visualized with the enhanced chemiluminescence detection system (ECL) as recommended by the manufacturer (Amersham Corp.).

Phosphatase treatment.

Twenty to sixty μg of whole cell extract were treated with 0.3 units of potato acidic phosphatase (Sigma) in a final volume of 30 μl PIPES buffer (10 mm PIPES pH 6.0, 0.5 mm PMSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) or 5 units of calf intestine alkaline phosphatase (Pharmacia) in 30 μl CIP buffer. The phosphatase inhibitor mix contained 10 mm NaF, 1.5 mm Na_2MoO_4, 1 mm β-glycerophosphate, 0.4 mm Na_3VO_4 and 0.1 μg/ml okadaic acid.

Subcellular localization of GFP-IRF-3 proteins.

To analyse the subcellular localization of wild type and mutated forms of IRF-3 proteins in uninfected and virus infected cells, the GFP-IRF-3 expression plasmids (5 μg) were transiently transfected into COS-7 cells by the calcium phosphate coprecipitation method. For virus infection, transfected cells were infected with Sendai virus (80 hemagglutinating units per mL for 2h) at 24h post transfection. GFP fluorescence was analyzed in living cells with a Leica fluorescence microscope using a 40x objective.

Electromobility Shift Assay.

Nuclear extracts were prepared from 293 cells at

different times after infection with Sendai virus (80HAU/ml). In som experiments, extracts were prepared from cells transfected with different IRF-3 expression plasmids, as indicated in individual experiments. Cells were washed in Buffer A [10 mM HEPES, pH 7.9; 1.5 mm $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol (DTT); and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and were resuspended in Buffer A containing 0.1% NP-40. Cells were then chilled on ice for 10 min before centrifugation at 10,000 g. Pellets were then resuspended in Buffer B (20mM HEPES, pH 7.9; 25% glycerol; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT; 0.5 mM PMSF; 5 μg/ml leupeptin; 5 μg/ml pepstatin; 0.5 mM spermidine; 0.15 mM spermine; and 5 μg/ml aprotinin). Samples were incubated on ice for 15 min before being centrifuged at 10,000 g. Nuclear extract supernatants were diluted with Buffer C (20 mM HEPES, pH 7.9; 20% glycerol; 0.2 mM EDTA; 50 mM KCl; 0.5 mM DTT; and 0.5 mM PMSF). Nuclear extracts were subjected to EMSA by using a 32P-labelled probe corresponding to the PRDIII region of the IFN-β promoter (5'-GGAAAACTGAAAGGG-3') or the ISRE region of the ISG-15 promoter (5'-GATCGGGAAAGGGAAACCGAAACTGAAGCC-3'). The resulting protein-DNA complexes were resolved by 5% polyacrylamide gel and exposed to X-ray film. To demonstrate the specificity of protein-DNA complex formation, 125-fold molar excess of unlabelled oligonucleotide was added to the nuclear extract before adding labelled probe.

Immunoprecipitation and Western analysis of CBP associated proteins.

Whole cell extract (300 μg) were prepared from either transfected or untransfected cells and precleared with 5 μl of preimmune rabbit serum and 20 μl of protein A-Sepharose beads (Pharmacia) for 1 h at 4°C. The extract was incubated with 10 μl of anti-CBP antibody A-22 (Santa Cruz) or 2 μl anti-myc antibody 9E10 (21) and 30 μl of protein A-Sepharose beads for 2-3 h at 4°C. Precipitates were washed 5 times with lysis buffer, eluted by boiling the beads 3 min in 1x SDS sample buffer. Eluted proteins were separated by SDS PAGE, transferred to Hybond transfer membrane. Membranes were incubated with anti-IRF-3 (1:3000) or anti-myc antibody 9E10 (1:1000). Immunocomplexes were detected by using a chemiluminescence-based system.

Virus induced phosphorylation of IRF-3 protein.

IRF-3 is expressed constitutively in various cells and its expression is not enhanced by viral infection or by IFN treatment. To investigate whether the IRF-3 protein is regulated by post-translational modification after virus infection, 293 cells were transiently transfected with an IRF-3 expression plasmid and subsequently infected with Sendai virus 24h later. In cells transfected with CMVBL vector alone, endogenous IRF-3 protein was easily detected using a polyclonal IRF-3 antibody and in cells transfected with the IRF-3 expression plasmid, IRF-3 protein levels were significantly increased (Fig.1, lanes 1 and 3). Interestingly, Sendai virus infection resulted in two

alterations in th expression of IRF-3: 1) an overall decrease in the amount of IRF-3 in transfected and control cells (Fig. 1, lanes 2 and 4) and the generation of a more slowly migrating form of IRF-3 (Fig. 1, compare lanes 1 and 2). In all experiments, the turnover of IRF-3 after virus infection was more pronounced with the endogenous protein than with the transfected proteins (see Fig.1, as well as others). Because the transfected proteins were driven by the CMV promoter, ongoing synthesis of transfected IRF-3 may partially obscure the turnover of IRF-3.

The kinetics of virus-induced modification of IRF-3 were characterized in a 293 cell line that expressed IRF-3 inducibly under the control of the tetracycline responsive promoter CMVt (25,26). Infection of this cell line (designated rtTA-IRF-3) with Sendai virus resulted in a decrease in the amount of IRF-3 between 12 and 24h after infection (Fig. 2A). Two forms of IRF-3 protein (designated I and II) were detected in uninfected cells (Fig. 2A, lane 1) and following virus infection, a third slowly migrating form of IRF-3 was also detected (Fig.2A, lanes 4-7). To determine whether the slowest form of IRF-3 was due to virus-induced phosphorylation (P-IRF-3), the different forms of IRF-3 were subjected to treatment *in vitro* with potato acidic phosphatase (PPA) or calf intestine alkaline phosphatase (CIP) and/or phosphatase inhibitors (Fig. 2B). These treatments did not affect the mobilities of forms I and II in uninfected cells (Fig. 2B, lanes 1-3). However, in rtTA-IRF-3 expressing 293 cells infected with Sendai virus

for 12h, an additional slowly migrating, presumably phosphorylated form of IRF-3 was also detected (Fig. 2B, lane 6); this form of IRF-3 completely disappeared following CIP or PPA treatment (Fig.2B, lanes 6 and 7) but was maintained in the presence of CIP/PPA when phosphatase inhibitors were also added to the reaction (Fig. 2B, lanes 5 and 8).

Mapping the IRF-3 phosphorylation sites.

A series of deletions of IRF-3 were generated to identify the virus-induced phosphorylation site(s) of IRF-3 (Fig. 3A). 293 cells were transiently transfected with IRF-3 deletion mutants and the virus mediated phosphorylation was measured by immunoblotting (Fig. 3B). The results indicated that a virus-induced phosphorylation of IRF-3 occurs at the C-terminal end of IRF-3 since the mutations that contained only the N-terminal part of IRF-3 protein (133, 240, 328, 357 or 394aa) were not phosphorylated (Fig. 3B). Full length and 407aa forms of IRF-3 were phosphorylated as a consequence of virus infection (Fig. 3B, lanes 1-4). C-terminal truncation of IRF-3 to a protein of 394 or 357aa removed the site(s) of inducible phosphorylation (Fig. 3B, lanes 5-8), although the shortened versions of forms I and II were still observed. Also in the IRF-3 Δ9-133 mutation (ΔN) which had the DNA binding, N-terminal amino acids (aa9 to aa133) removed, both virus induced phosphorylation of IRF-3 and the differential migration of the shortened forms I and II were easily detected (Fig. 3B, lanes 9 and 10). Degradation of the endogenous forms of IRF-3 by virus infection was also detected in this experiment (compare Fig. 3B, lanes 7 and 9

with lanes 8 and 10).

Thus, by deletion analysis, a phosphorylation domain of IRF-3 protein was localized to the region -ISNSHPLSLTSDQ- between amino acids 395 and 407. Point mutations in the several putative Ser and Thr phosphorylation residues within this region were generated in the full length protein and the Δ9-133 (ΔN) protein (Fig. 4A). In the IRF-3 cDNA encoding these proteins, the Ser-396/Ser398/Ser-402/Thr-404/Ser-405 residues were replaced by alanine (5A), as were the three residues Ser-402/Thr-404/Ser-405 (3A) and the two residues Ser-396/Ser-398 (2A). Transfection of these plasmids into 293 cells and subsequent virus infection revealed that full length wild type IRF-3 was phosphorylated (Fig. 4B, lanes 4 and 8), whereas the IRF-3 proteins containing 2A and 5A mutations were no longer phosphorylated in virus infected cells (Fig. 4B, lanes 6 and 10). Interestingly, IRF-3-3A was also very weakly phosphorylated as a consequence of virus infection, thus implicating Ser-402/Thr-404/Ser-405 as potential secondary sites of phosphorylation. Using the ΔN IRF-3 protein and the relevant point mutations, phosphorylation was detected with ΔN (Fig. 4B, lane 12) but not with ΔN-2A and ΔN-5A (Fig. 4B, lanes 14 and 18); likewise, ΔN-3A displayed very weak phosphorylation (Fig. 4B, lane 16).

These experiments thus implicate Ser-396 and Ser-398 as critical sites of virus-induced phosphorylation of IRF-3; however, Ser-402/Thr-404/Ser-405 residues also

contribute to the observed phosphorylation, since the migration of phosphorylated ΔN-3A is significantly faster than ΔN and the phosphorylation level is decreased (Fig. 4B, lanes 12 and 16). Another study suggested the involvement of the Ser residues at aa385 and 386 as potential phosphoacceptor sites (67); in studies with the S385A/S386A mutation, applicant found no evidence for inducible phosphorylation at these sites. However, since these sites represent consensus sites for CKI and CKII, constitutive phosphorylation is a possibility.

IRF-3 phosphorylation induces cytoplasmic to nuclear translocation of IRF-3.

Initial studies indicated that IRF-3 was localized in the cytoplasm of uninfected cells (67); to investigate the role of phosphorylation on IRF-3 localization, wild type and point mutated forms of IRF-3 were linked to green fluorescent protein (GFP), transfected into COS-7 cells and examined for Sendai virus induced changes in subcellular localization (Fig. 5). In uninfected cells, GFP-IRF-3 localized exclusively to the cytoplasm; Sendai virus infection resulted in translocation of IRF-3 to the nucleus within 8h in 90-95% of the cells (Fig. 5A and B). Mutation of the Ser/Thr cluster in GFP-IRF-3(5A) completely abrogated virus-induced cytoplasmic to nuclear translocation (Fig. 5, C and D). Interestingly, the substitution of the Ser/Thr cluster with the phosphomimetic Asp in GFP-IRF-3(5D) likewise altered subcellular localization. IRF-3(5D) localized both to the nucleus and cytoplasm in uninfected cells (Fig. 5E), while

virus infection resulted in an intense nuclear pattern of IRF-3(5D) fluorescence (Fig. 5F). Point mutation of a putative nuclear export signal in IRF-3, the L145A/L146A modification - termed IRF-3(NES) - also changed subcellular localization of IRF-3. In uninfected cells, GFP-IRF-3(NES) was localized to the nucleus and cytoplasm, with a homogeneous, extra-nucleolar pattern of nuclear staining. After virus infection, GFP-IRF-3(NES) localized to the nucleus with an intense speckled pattern of nuclear fluorescence in greater than 95% of the cells, suggesting that IRF-3(NES) may be trapped in the nucleus associated with the nuclear pore complex.

Transactivation of PRDI/PRDIII and ISRE promoters by IRF-3.

Next, the capacity of IRF-3 to regulate gene expression was analysed by transient transfection in human 293 and murine NIH3T3 cells using the IFNβ and ISG-15 promoters in reporter gene assays. Expression of NF-κB RelA(p65), IRF-1 and IRF-3 alone minimally induced IFNβ promoter activity between 3 to 4 fold (Fig. 6A and B), as shown previously (24,56,61). Introduction of the C-terminal point mutants - IRF-3(2A), IRF-3(3A) IRF-3(5A) - reduced the low transactivation capacity of IRF-3 to control levels (Fig. 6A). Interestingly, deletion of the C-terminal 20aa of IRF-3 to IRF-3(407) stimulated IFNβ activity about 6 fold, indicative of the removal of an inhibitory domain in IRF-3. However, further deletion to 394, 357 or 240 abrogated transactivation potential (Fig. 6A). Mutation of the NES element was not sufficient to stimulate IFNβ activity.

Strikingly, the substitution of the Ser/Thr cluster at aa397-405 in IRF-3 with the phosphomimetic Asp generated a very strong, constitutive transactivator protein that alone stimulated the IFNβ promoter 90 fold.

As shown previously, high level induction of the IFNβ promoter requires synergistic activation by NF-κB and IRF proteins (24,61). To analyse the properties of IRF-3 in synergistic activation of the IFNβ promoter, co-expression studies were performed using RelA(p65) expression plasmid and different wild type and mutant forms of IRF-3 (Fig. 6B). Co-expression of RelA and IRF-1 or RelA and IRF-3 stimulated IFNβ-CAT activity by 20-25 fold. IRF-3(407) and RelA(p65) stimulated IFNβ activity about 40 fold, supporting the idea of the removal of an inhibitory domain in IRF-3, whereas both the IRF-3(394) and the IRF-3(NES) failed to synergise with RelA in the activation of the IFNβ promoter. RelA and IRF-3(NES) produced a relatively weak 8 fold induction of IFNβ expression, indicating that nuclear localization is not sufficient for IRF-3 activation. The combination of RelA and IRF-3(5D) produced an 80 fold stimulation of IFNβ promoter activity (Fig. 6B); together with the above data, IRF-3(5D) alone appears to be capable of full stimulation of the IFNβ promoter and further synergy with RelA is not observed (compare Fig. 6A and B). Surprisingly, IRF-3(5A) and RelA produced a 30 fold stimulation, suggesting that 5A can still synergise with RelA, despite mutation of the Ser/Thr cluster.

The transactivation potential of IRF-3 was also analysed using the ISG-15 promoter, an ISRE containing

regulatory element (Fig. 6C). As shown previously (2), and above for the IFNβ promoter, IRF-3 alone weakly activated the ISG-15 promoter; in the context of this regulatory element, IRF-3 was weaker than IRF-1, which produced a 9 fold stimulation. Again deletion of the C-terminal 20aa of IRF-3 generated a protein that stimulated gene expression; with the ISG-15 promoter, a 12 fold induction was observed; IRF-3(394) and IRF-3(357) did not stimulate gene expression but rather slightly repressed ISG-15. Again remarkably, IRF-3(5D) produced a 50 fold induction of the ISG-15 promoter (Fig. 6C), thus demonstrating that substitution of the Ser/Thr sites with the phosphomimetic Asp generated a constitutively active form of IRF-3 that functioned as a very strong activator of promoters containing PRDI/PRDIII or ISRE regulatory elements.

Inhibition of IRF-3 degradation.

Another consequence of virus infection is the degradation of the IRF-3. Since phosphorylation of proteins is functionally associated with the process of protein degradation via the ubiquitin-dependent proteasome pathway (53,57,60), the effect of proteasome inhibitors on virus-induced turnover of IRF-3 was examined. In cells transfected with the ΔN and ΔN5A forms of IRF-3, virus induced degradation of full length (endogenous) forms of IRF-3 (Fig. 7A, lanes 1 and 4) and the truncated ΔN (Fig. 7B, lanes 1 and 4) was detected. Addition of the protease inhibitor calpain inhibitor I or the proteasome inhibitor MG132 blocked virus-induced IRF-3 degradation (Fig. 7A and

7B, lanes 4-6). Particularly with the ΔN protein, the accumulation of the phosphorylated form of ΔN was also detected in virus infected cells (Fig. 7B, lanes 5 and 6), suggesting that phosphorylation of IRF-3 may represent a signal for subsequent degradation by the proteasome pathway. To confirm this idea, the 5A point mutated form of IRF-3 was analysed; the IRF-3-ΔN5A protein was resistant to virus induced degradation (Fig. 7C, lanes 1 and 4); no further stabilization of IRF-3-ΔN5A occurred with calpain inhibitor I or MG132 addition and no phosphorylated IRF-3 was detected (Fig. 7C, lanes 4-6). These experiments demonstrate that virus dependent phosphorylation of the C-terminal of IRF-3 represents a signal for subsequent proteasome mediated degradation.

Interaction between IRF-3 and CBP in virus infected cells.

To examine the possibility that IRF-3 associated with the co-activator CBP/p300 (Fig. 8A) following Sendai virus infection, CBP was immunoprecipitated from virus-infected cells with anti-CBP antibody; an immunoblot for IRF-3 revealed that IRF-3 was co-precipitated from virus-infected cells but not from uninfected cells (Fig. 8B, lanes 2 and 3). This interaction was observed clearly in cells co-transfected with the IRF-3 expression plasmid (Fig. 8B, lane 3) but was not seen when the immunoprecipitation was performed with pre-immune serum (Fig. 8B, lane 7). The endogenous IRF-3 also co-precipitated from virus-infected cells (Fig. 8B, lane 1). However, mutation of the Ser/Thr residues identified as the virus inducible phosphorylation

sites abrogated the association of IRF-3 with CBP. In particular, IRF-3(2A) and IRF-3(5A) were detected in whole cell extract immunoblot but not in the CBP immunoprecipitate (Fig. 8B, compare lanes 4 and 6 with lanes 11 and 13). With the IRF-3(3A) mutant, interaction with CBP was still observed (Fig. 8B, lane 5). The high background in all lanes represents secondary antibody reactivity with rabbit IgG from the immunoprecipitation. Immunoblot analysis of the whole cell extracts revealed that phosphorylated IRF-3, as well as forms I and II were present in virus infected cells (Fig. 8B, lane 10) and in cells transfected with 2A, 3A and 5A the forms I and II were observed but not the phosphorylated form of IRF-3 (Fig. 8B, lanes 11-13).

CBP has several domains that bind transcription factors, designated CBP1, CBP2, and CBP3 respectively (Fig. 8A, reviewed in (28)). To determine which domain of CBP interacts with IRF-3, the three specific subdomains were myc-tagged at the 5' end by subcloning into the pCDNA3 vector (Fig. 8A). 293 cells were co-transfected with these myc-tagged CBP expression plasmids together with the IRF-3 ΔN (Δ9-133) expression plasmid. At 24h after transfection, cells were infected with Sendai virus, co-immunopreciptated with anti-myc antibody 16h later (21) and then immunoblotted for IRF-3. Endogenous IRF-3 and transfected IRF-3 ΔN proteins co-precipitated with CBP-3 from virus-infected cells but not from uninfected cells (Fig. 8C, lane 6). In cells co-transfected with CBP-1 and CBP-2, no endogenous or transfected ΔN IRF-3 was detected (Fig. 8C, lanes 1-4).

Immunoblot analysis of the whole-cell extracts revealed that all three myc-tagged CBP proteins were efficiently expressed in uninfected and virus infected cells (Fig. 8D). These results demonstrate that IRF-3 binds to the C-terminal domain of CBP in virus infected cells and interaction with CBP requires Ser-396/Ser-398 phosphorylation of IRF-3 but not at Ser-402/Thr-404/Ser-405.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A phosphorylated form of IRF-3 wherein Ser-396 and Ser-398 residues are phosphorylated.

2. IRF-3 according to claim 1 wherein one or more of Ser-402, Thr-404 and Ser-405 are phosphorylated.

3. A mutant form of IRF-3 wherein one or more of Ser-396, Ser-398, Ser-402, Thr-404 and Ser-405 are substituted by phosphomimetic Asp or phosphomimetic Glu.

4. Use of the IRF-3 according to any one of claims 1 through 3 to activate one or more genes selected from interferon genes and cytokine genes.

5. Use of the IRF-3 according to any one of claims 1 through 3 to treat virus infections.

Figure 1

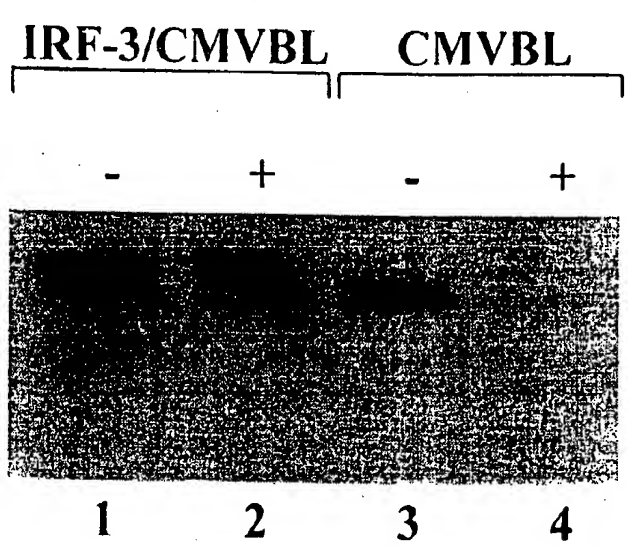

Figure 2

A


Sendai virus (h) - 4 8 12 16 20 24
P
II
I
1 2 3 4 5 6 7

B

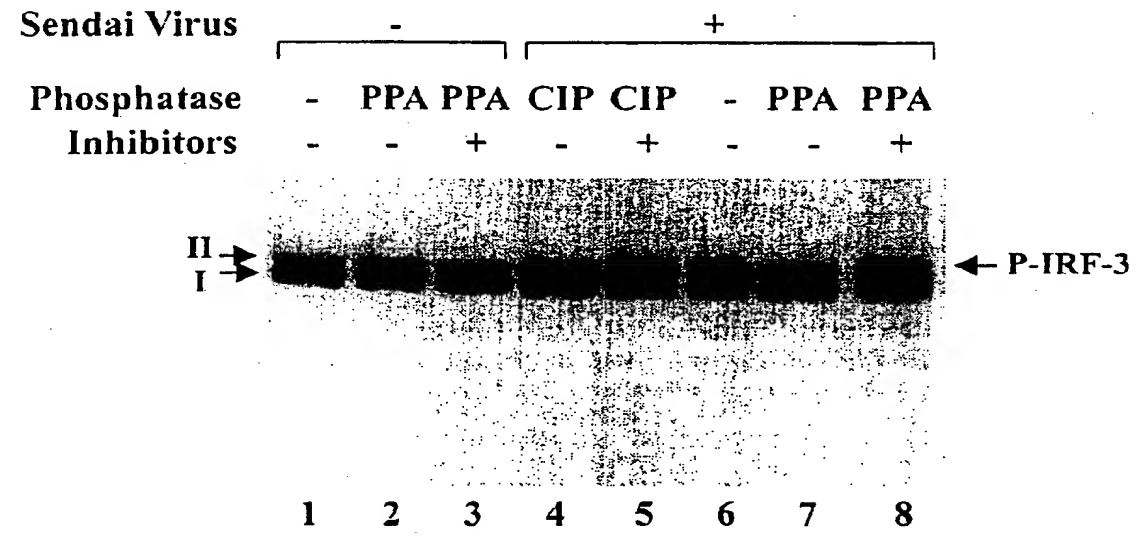
Sendai Virus - +
Phosphatase - PPA PPA CIP CIP - PPA PPA
Inhibitors - - + - + - - +
II
I
P-IRF-3
1 2 3 4 5 6 7 8

Figure 3A

A

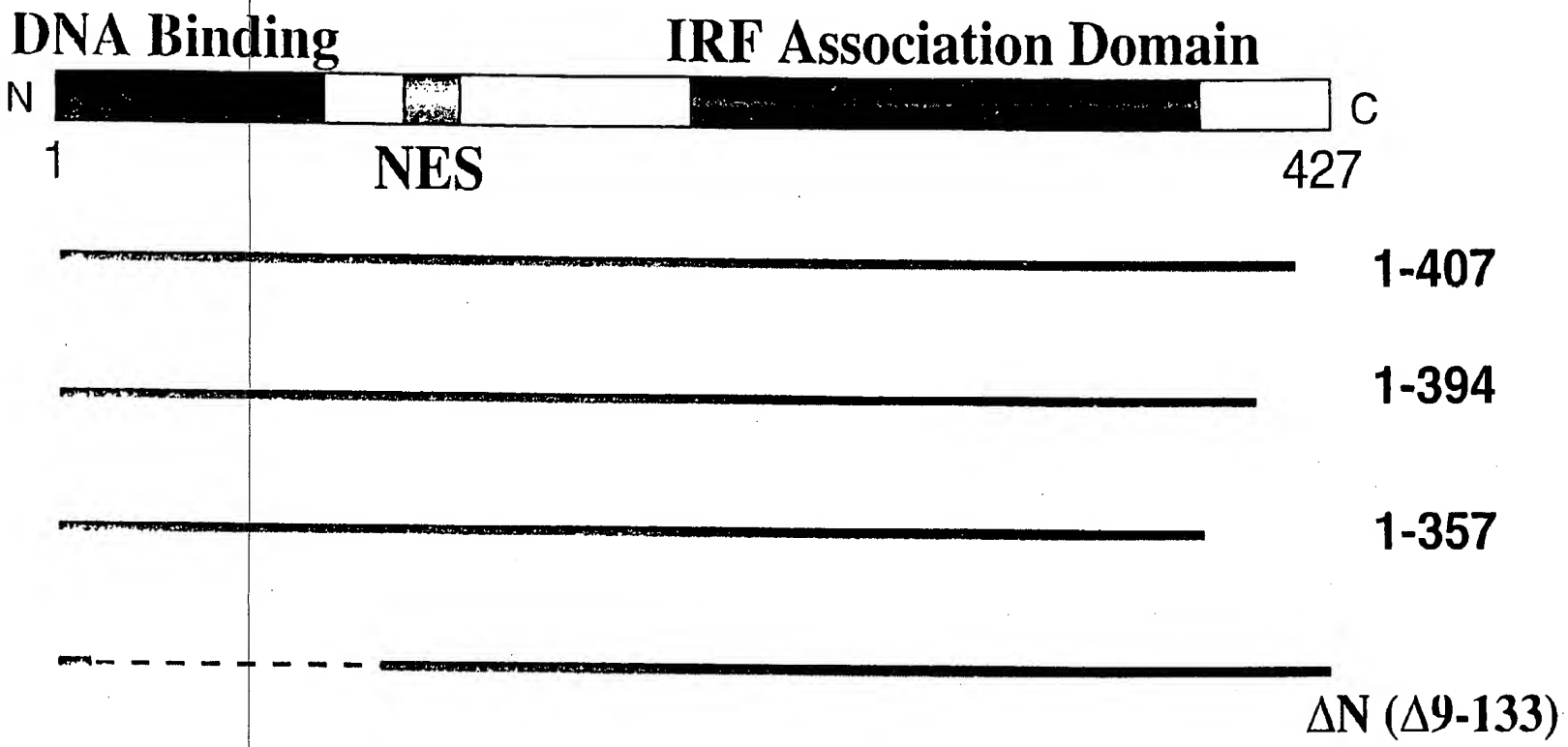

Figure 3B

B

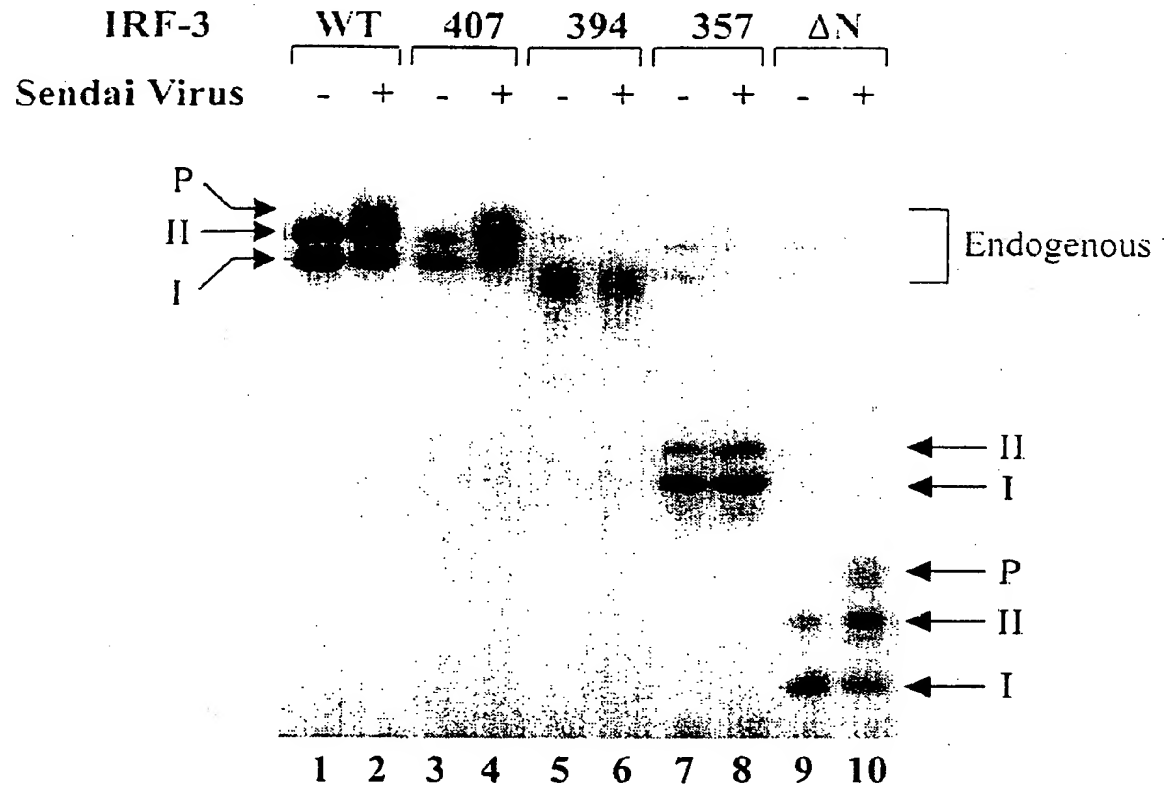
IRF-3
WT
407
394
357
ΔN
Sendai Virus
- + - + - + - + - +
P
II
I
Endogenous
II
I
P
II
I
1 2 3 4 5 6 7 8 9 10

Figure 4A

A

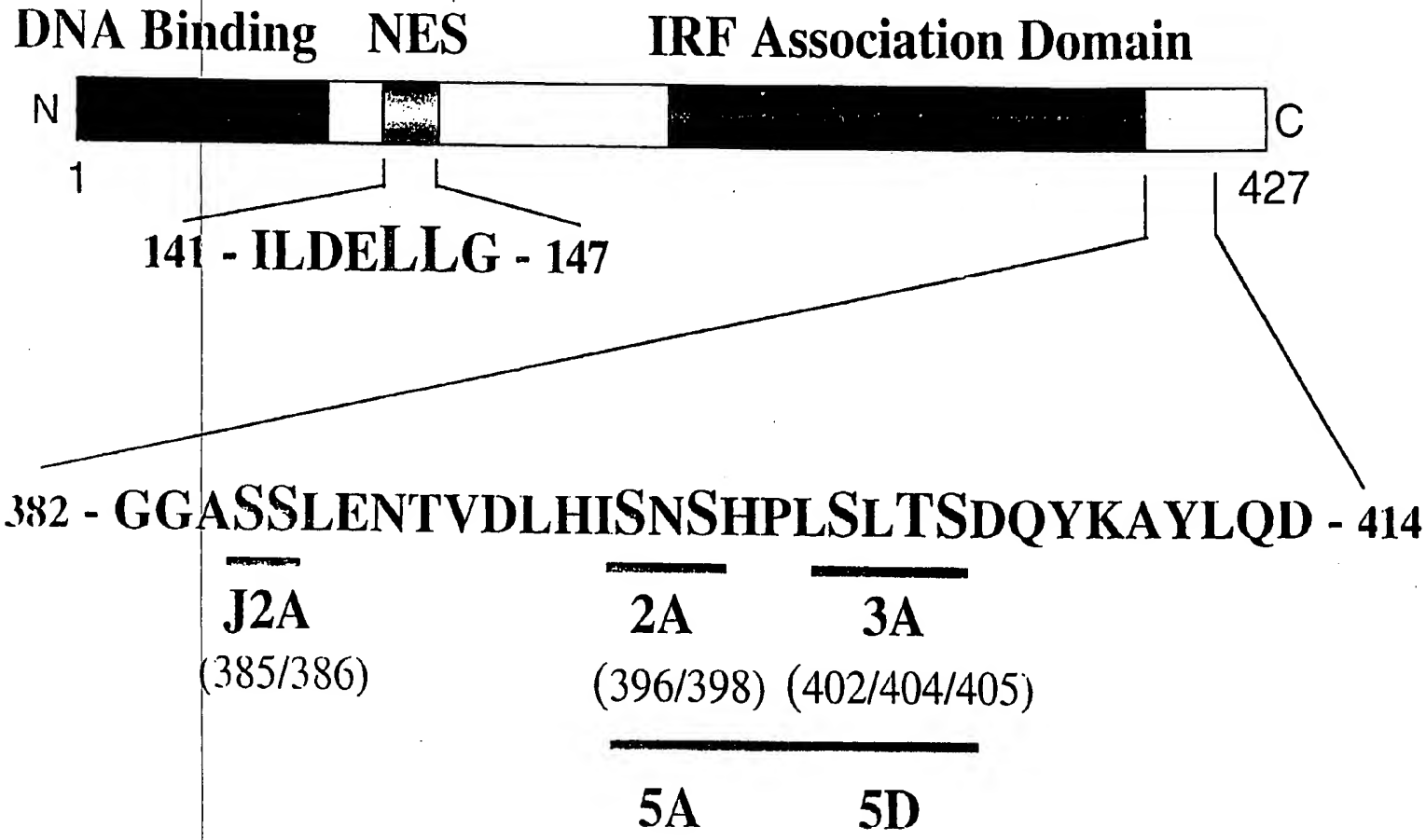

Figure 4B

B

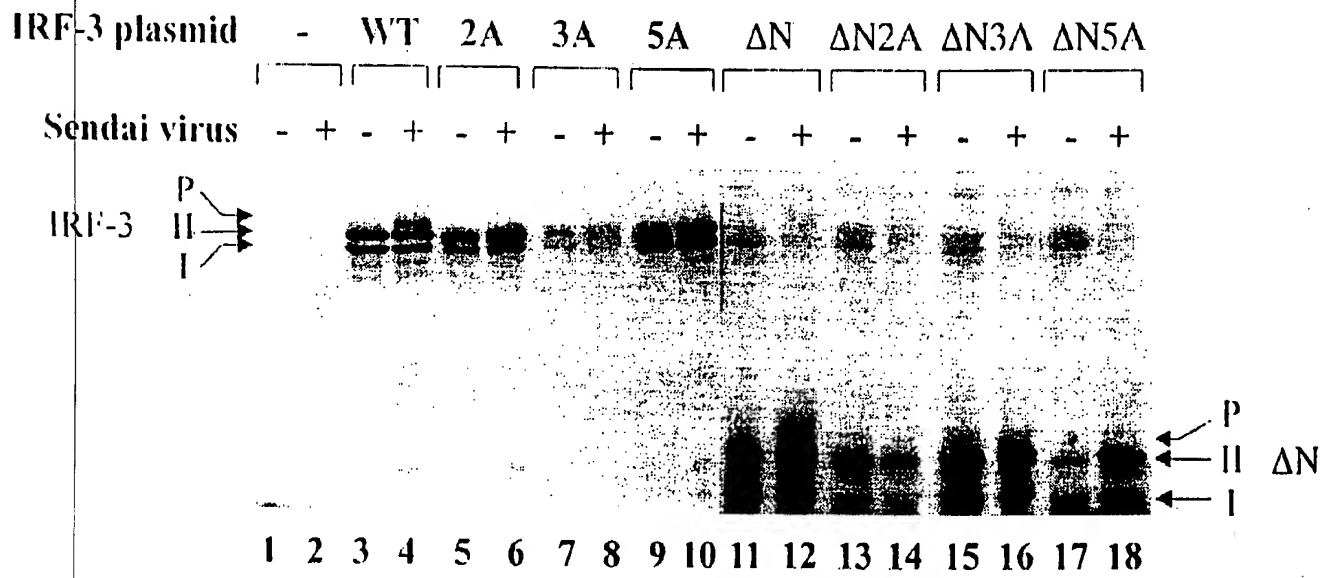

Figure 5

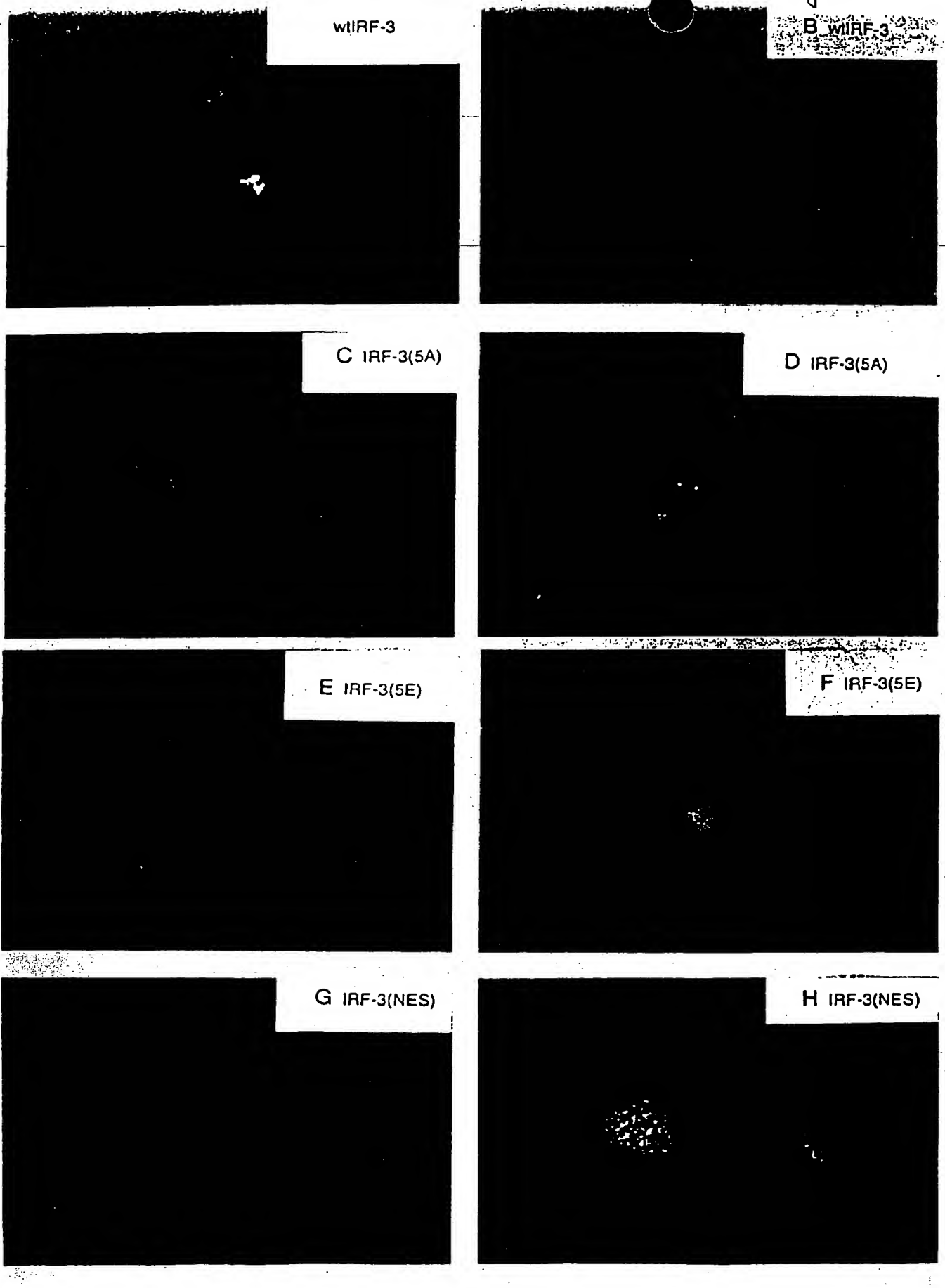
wtIRF-3
B wtIRF-3
C IRF-3(5A)
D IRF-3(5A)
E IRF-3(5E)
F IRF-3(5E)
G IRF-3(NES)
H IRF-3(NES)

Figure 6A

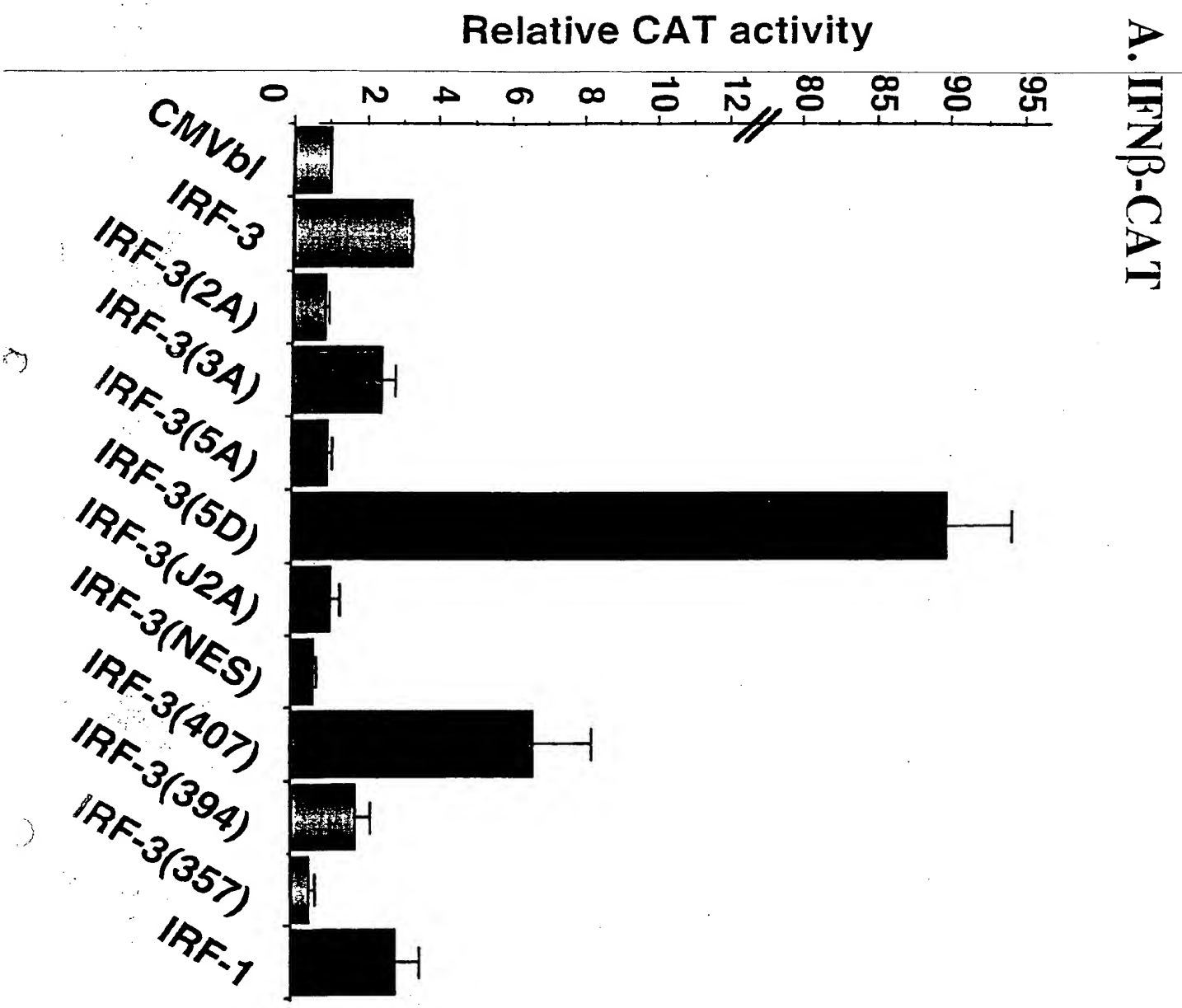
A. IFNβ-CAT
Relative CAT activity
0
2
4
6
8
10
12
80
85
90
95
CMVbl
IRF-3
IRF-3(2A)
IRF-3(3A)
IRF-3(5A)
IRF-3(5D)
IRF-3(J2A)
IRF-3(NES)
IRF-3(407)
IRF-3(394)
IRF-3(357)
IRF-1

Figure 6B

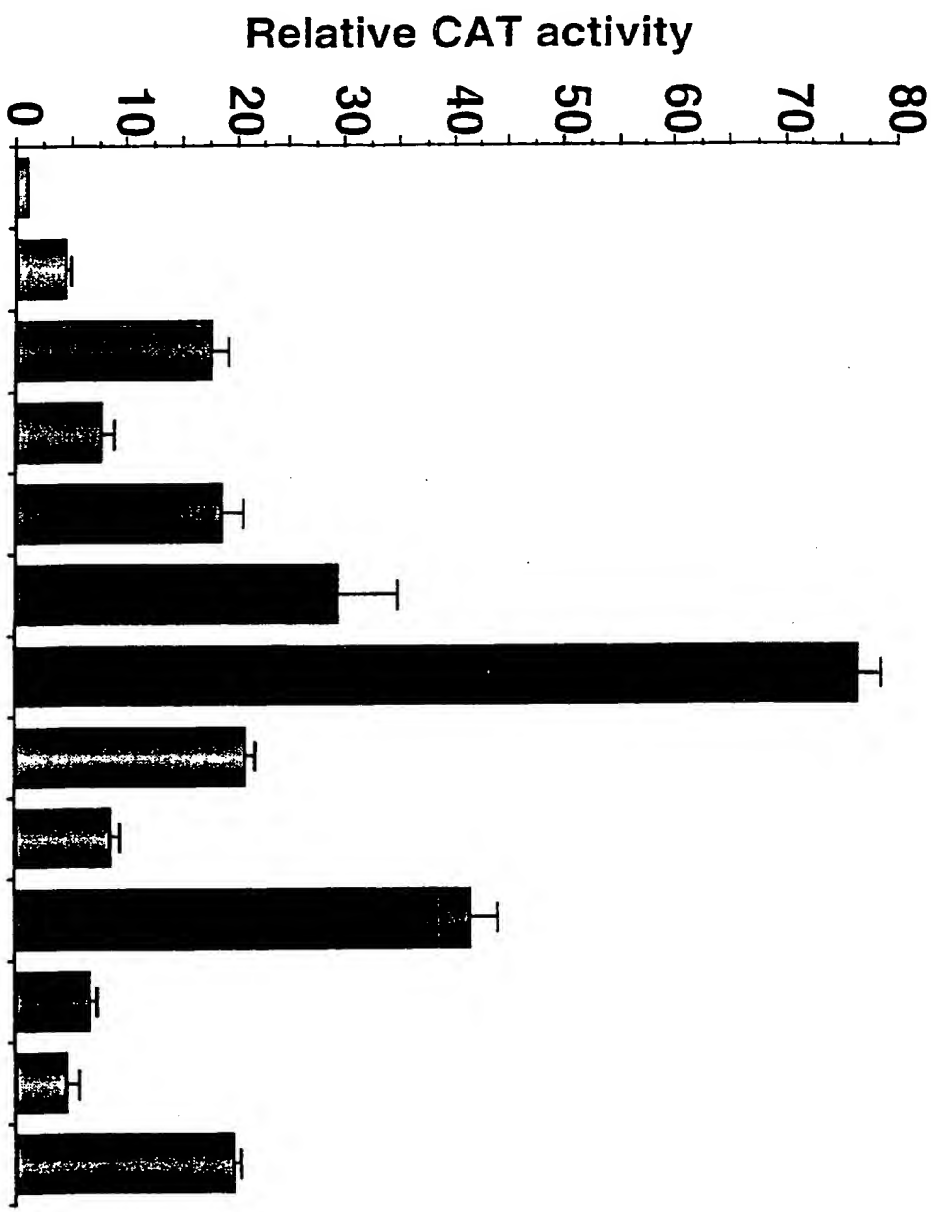
B. IFNβ-CAT
Relative CAT activity
0
10
20
30
40
50
60
70
80
RelA - + + + + + + + + +
IRF-3 - - WT 2A 3A 5A 5D J2A NES 407 394 357 IRF-1

Figure 6C

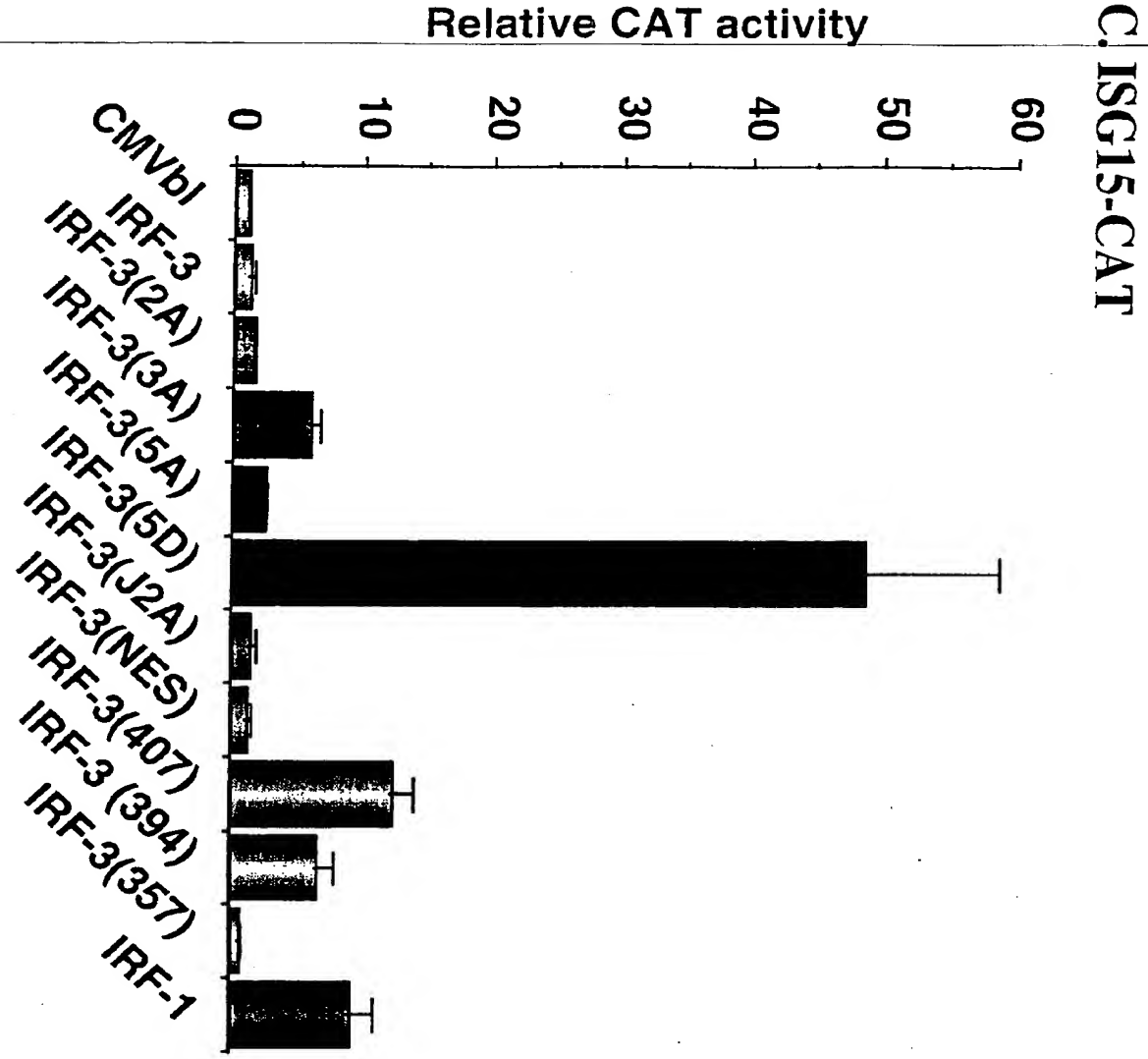
C. ISG15-CAT
Relative CAT activity
0
10
20
30
40
50
60
CMVbI
IRF-3
IRF-3(2A)
IRF-3(3A)
IRF-3(5A)
IRF-3(5D)
IRF-3(J2A)
IRF-3(NES)
IRF-3(407)
IRF-3 (394)
IRF-3(357)
IRF-1

Figure 7

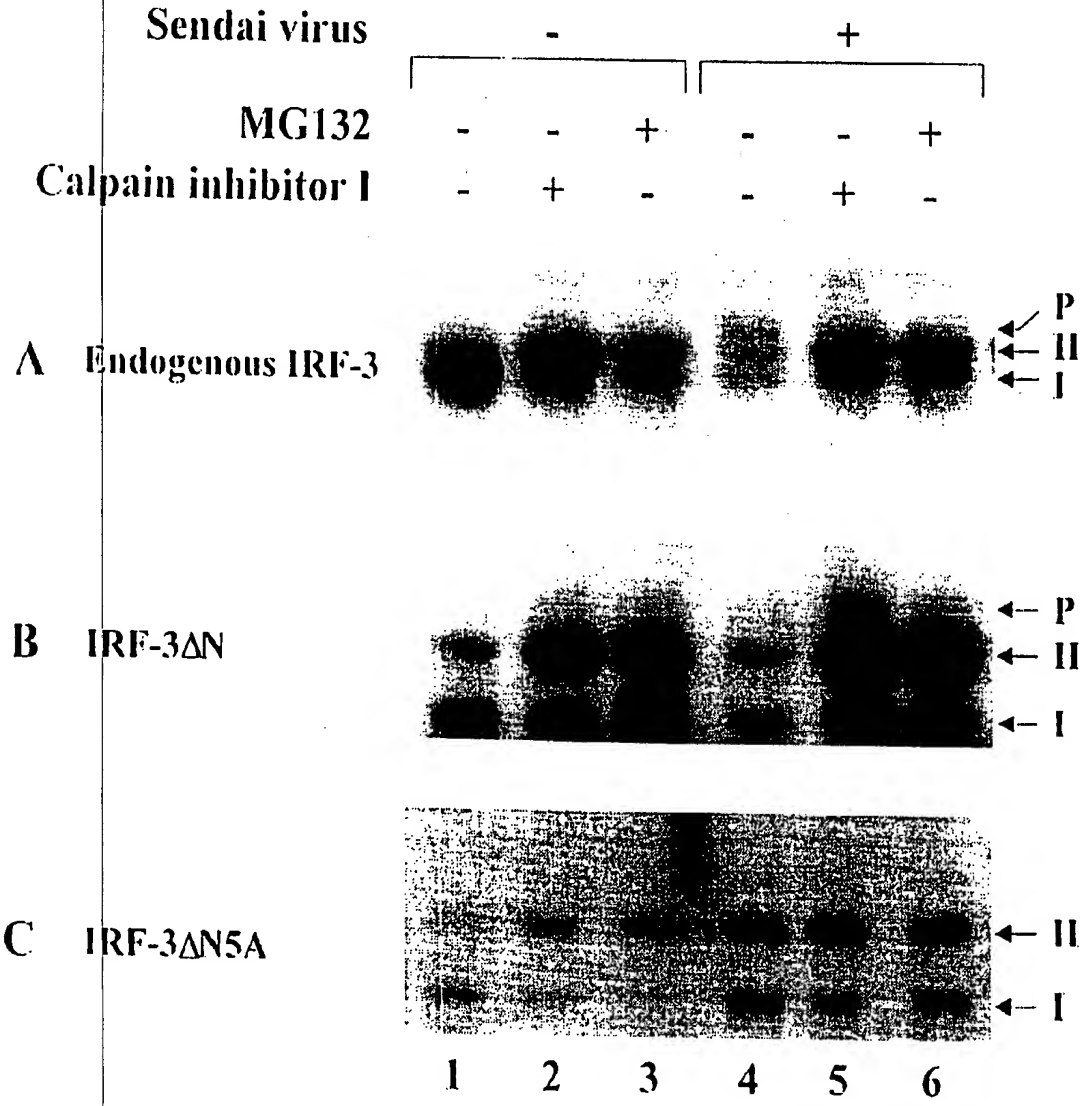

Figure 8A

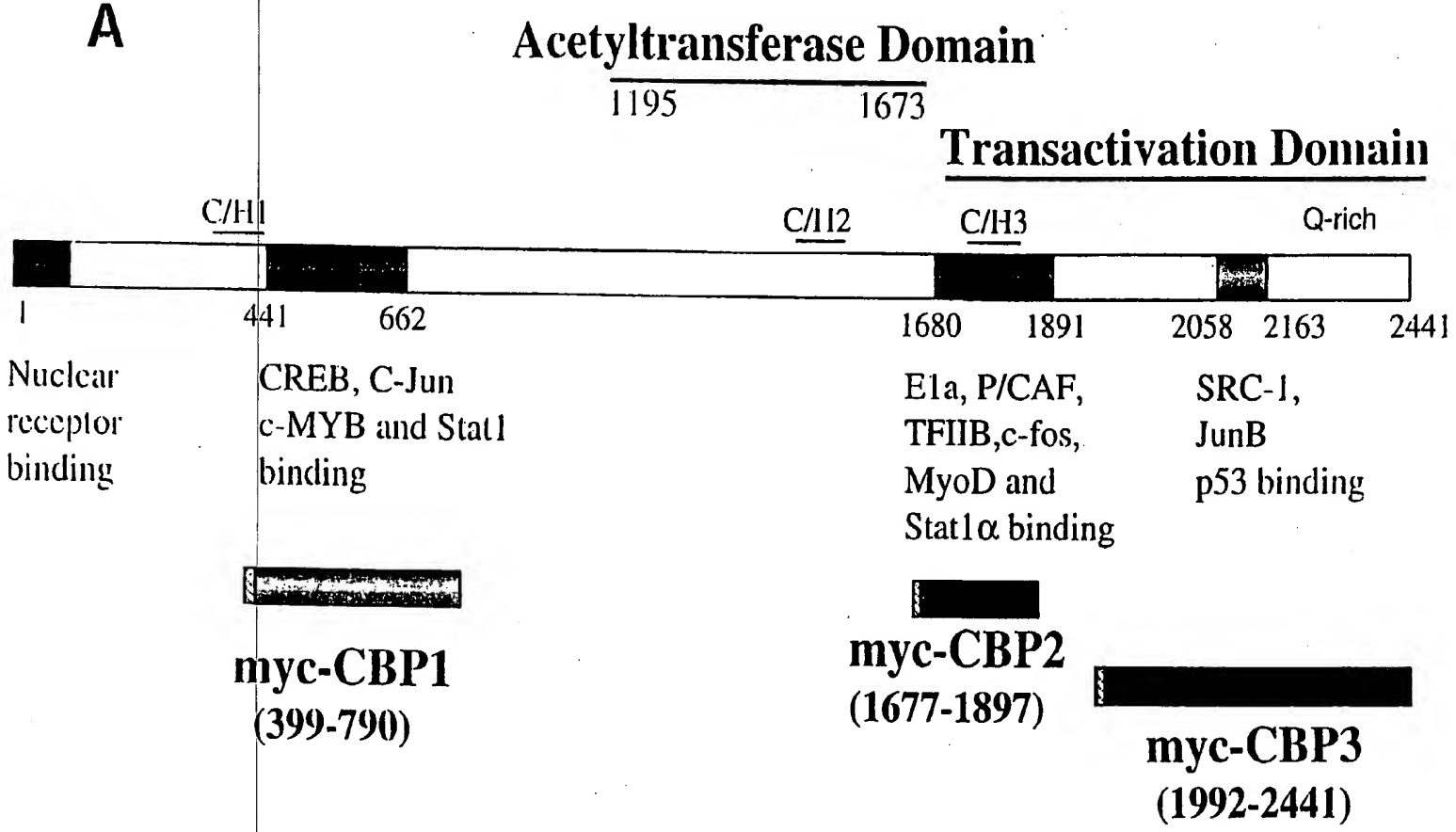

Figure 8B

B

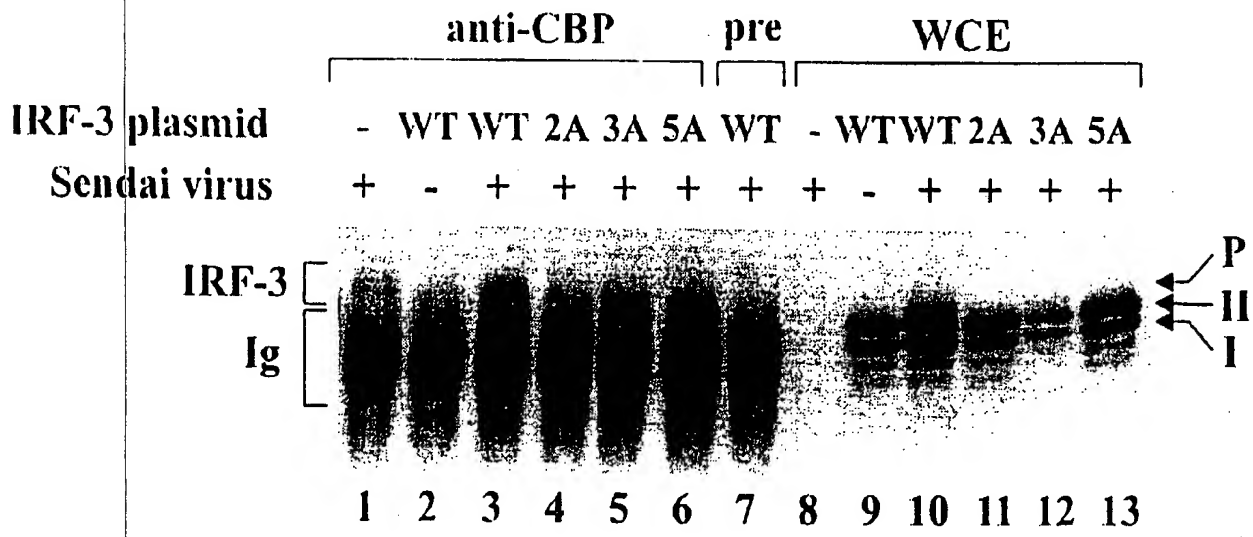

Figure 8C